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The Fine Structure of the Amebocyte in the Blood of *Limulus polyphemus*. II. The Amebocyte Cytoskeleton: A Morphological Analysis of Native, Activated, and Endotoxin-Stimulated Amebocytes

FERN TABLIN[1] AND JACK LEVIN[2]

[1]*Department of Anatomy, School of Veterinary Medicine, University of California, Davis, California 95616, and* [2]*Departments of Laboratory Medicine and Medicine, University of California School of Medicine, San Francisco, California 94143 and the Veterans Administration Medical Center, San Francisco, California 94121*

Abstract. This study evaluates the structural organization of the cytoskeleton in native, activated, and endotoxin-activated amebocytes of the horseshoe crab, *Limulus polyphemus*. The discoid shape of native amebocytes appears to be maintained by a three-part arrangement consisting of a microtubule marginal band in a two-dimensional plane, an extensive actin-membrane array, and three-dimensional cortical actin array. This arrangement is disrupted during amebocyte activation in the absence of endotoxin, as the cortical actin array becomes diffuse and the cells form many pseudopodia. However, under these conditions the marginal band remains intact and continues to be located at the periphery of these cells. Amebocytes activated by bacterial endotoxin display a different cytoskeletal arrangement. After exposure to endotoxin, the cells displace their entire cortical actin array and marginal band to a more centralized location surrounding the nucleus.

Introduction

The amebocytes of the horseshoe crab, *Limulus polyphemus*, provide a useful model for the study of hemostasis and thrombosis. In *Limulus*, the amebocyte is the only type of circulating blood cell and is characterized by its large cytoplasmic granules, which contain the factors necessary for blood coagulation (Levin and Bang, 1964a, b, 1968; Murer *et al.*, 1975). Amebocytes are sensitive to the presence of bacterial endotoxins, which produce aggregation, activation, and degranulation (Bang, 1956, 1979; Levin and Bang, 1964a; Ornberg and Reese, 1981; Armstrong and Rickles, 1982). Amebocytes are also motile cells which can be observed in the abdominal spines (Bang, 1979) or isolated gill leaflets of young animals (Loeb, 1928; Armstrong, 1979). Studies of amebocytes treated with cytochalasin D have shown an inhibition of motility (Armstrong, 1979). This has led investigators to suggest that an actomyosin system is responsible for the generation of motile forces. Morphologic studies of activated amebocytes have demonstrated bundles of thin filaments within filopodia of motile cells (Armstrong, 1985). Studies of unactivated amebocytes have revealed a marginal band of microtubules (MB) present at the periphery of the cells (Dumont *et al.*, 1966; Nemhauser *et al.*, 1980). It has been suggested that the MB acts to stabilize the discoid shape of the amebocyte (Cohen and Nemhauser, 1985). In this report, we describe the cytoskeleton in native amebocytes and its subsequent rearrangement after activation and granule release in the absence or presence of bacterial endotoxin.

Materials and Methods

Horseshoe crabs (*Limulus polyphemus*) were obtained from the Department of Marine Resources, Marine Biological Laboratory, Woods Hole, Massachusetts.

Unactivated (native) amebocytes were obtained by

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cardiac puncture with a sterile, endotoxin-free 18 gauge needle. Blood was allowed to drip directly into fixative or was aspirated into a sterile, endotoxin-free syringe and concomitantly combined with fixative. Activated amebocytes were generated by taking hemolymph directly into a sterile, endotoxin-free syringe and allowing the cells to aggregate and adhere to the plastic surface. After activation was apparent, as demonstrated by visible aggregation of amebocytes, activated amebocytes were carefully discharged from the syringe directly into fixative. Endotoxin-activated cells were prepared by taking hemolymph directly into syringes containing endotoxin (*E. coli* B, 055:B5, Difco Laboratories, Detroit, Michigan, in 3% NaCl). The ratio of hemolymph to endotoxin-containing saline was 9:1; the final concentration of endotoxin was 1 μg/ml.

All cells were fixed with 2.5% glutaraldehyde and 0.5% tannic acid in 100 m*M* phosphate buffer, pH 7.0, at room temperature. Samples were washed in 100 m*M* phosphate buffer, and post-fixed in 2% osmium with 2% potassium ferrocyanide in 100 m*M* phosphate buffer. An additional population of native amebocytes also was fixed in 2% glutaraldehyde with 50 m*M* lysine (Boyles *et al.*, 1985) and stained with 1% osmium. All amebocytes were dehydrated and embedded in Epon 812. Thin sections were stained with saturated uranyl acetate (in 70% ethanol) and lead citrate and examined on a Hitachi 600 transmission electron microscope.

Nuclear profiles were measured by the use of concentric circle grids, and axial ratios (Q) were calculated according to the method of Elias and Hyde (1983). Axial ratios are defined as the quotient length/width. Determination of nuclear shape was made by the use of axial ratios, as described by Elias and Hyde (1983). These measurements were undertaken to allow us to evaluate whether cytoskeletal changes reflected direct cytoplasmic interactions or were secondary to an increase or decrease in the size of the nucleus, resulting in a subsequent displacement of any particular cytoplasmic structure.

Results

Native amebocytes

The native amebocyte is a discoid cell with a centrally located nucleus and prominent cytoplasmic granules (Fig. 1). The cytoskeleton can be divided into two major filamentous elements: thin (5–7 nm) filaments (presumably actin) and microtubules (25–28 nm).

Thin filaments were observed in several different arrays. There was a prominent cortical band of actin filaments, some of which were associated with the plasma membrane (Fig. 2A). A diffuse network of thin filaments also was located throughout the remainder of the cytoplasm. Polyribosomes were frequently found in association with these thin filaments. The actin array was best seen in cells that had been fixed in the presence of lysine, according to the method of Boyles *et al.* (1985). On rare occasions, wavy 10-nm filaments were seen in the cytoplasm. These filaments were often located near the cortical band of thin filaments, and may represent a minor population of intermediate filaments or aggregates of thin filaments (Fig. 2A).

Microtubules were present in the form of a marginal band, as has been observed by other investigators (Fawcett and Witebsky, 1964; Dumont *et al.*, 1966; Nemhauser *et al.*, 1980; Ornberg and Reese, 1981). The marginal band was located internally to the cortical actin array. Projections or "arms" were observed in association with microtubules in both longitudinal and cross-section. These projections often appeared to extend from one microtubule to another (Fig. 2B).

Native amebocytes contained a large population of granules that were randomly distributed in the cytoplasm, but which were always located internally to the marginal microtubule band and cortical actin array.

Activated amebocytes

Activated amebocytes, no longer discoid in shape, often developed multiple pseudopodia. Fine filopodia rarely were seen extending from the distal ends of pseudopodia.

The organization of actin in the activated amebocyte varied depending upon its location within the cell. The cortical actin filaments were diminished in number and no longer formed a distinct array. Small numbers of thin filaments (microfilaments) remained in association with the plasma membrane. Actin arrays, both within the body of the cell and within pseudopodia, were diffuse, with filaments forming a fine interconnecting meshwork (Fig. 3). This internal meshwork was similar to that observed in the cytoplasm of native amebocytes. Some areas contained microspikes, in which thin filaments formed discrete bundles, which often extended back into the body of the cell (Fig. 4). Fine filopodia were rarely observed; however, when present, they too contained bundles of microfilaments.

Microtubules were present as a marginal band (MB) within the body of the cells (Fig. 3). However, MB's also were observed within pseudopodia. This was detected primarily when a pseudopodium was extended within the same plane as the marginal band. (Data not shown.) Individual microtubules rarely were seen in pseudopodia.

Examinations of activated amebocytes revealed that many cells were undergoing exocytosis of granules. This

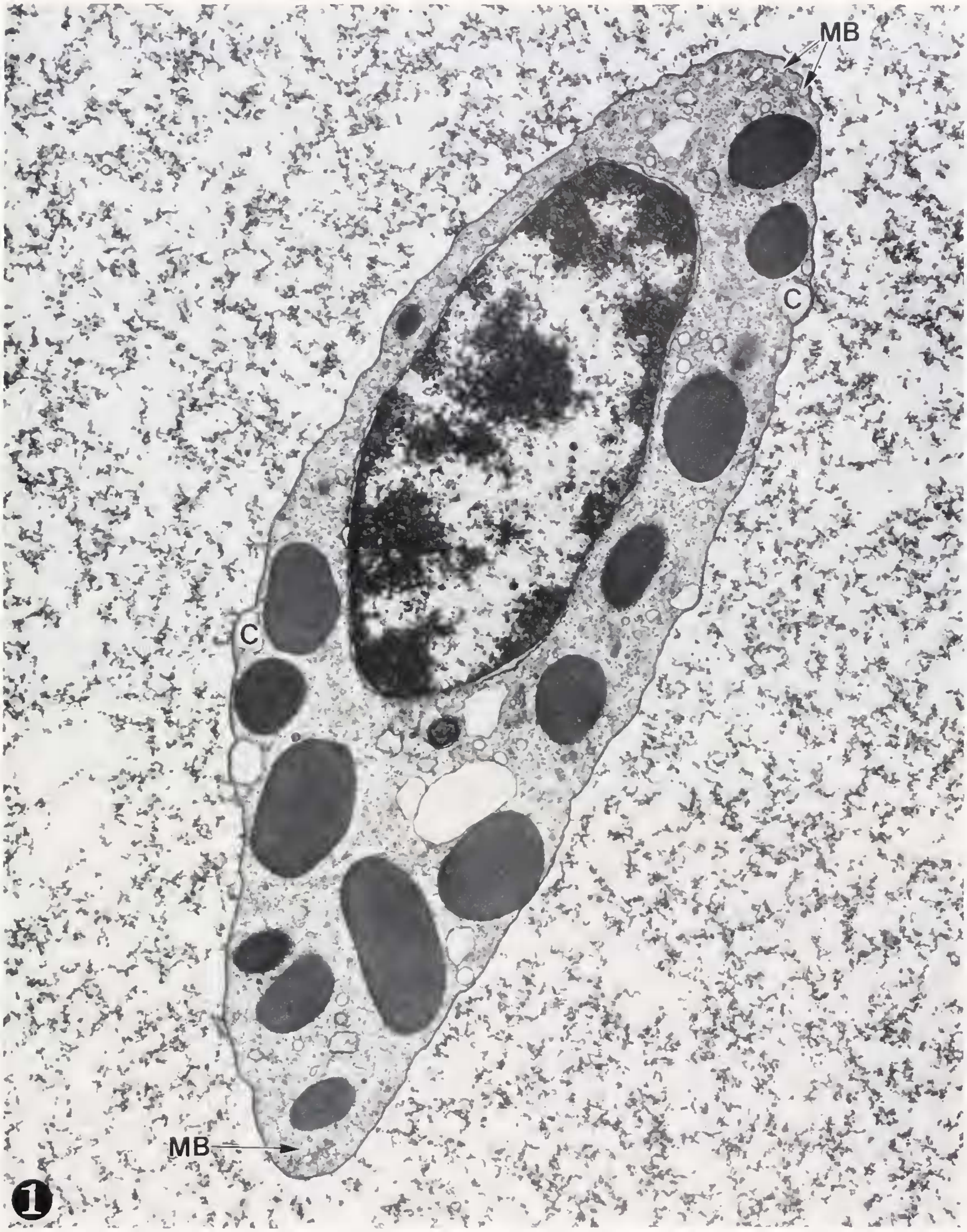

Figure 1. Native amebocytes are discoid in shape. The marginal band (MB) of microtubules is present in cross-section, at either end of the cell. Small cisternae (C) may represent intracellular calcium stores. Glutaraldehyde and tannic acid fixation (see Materials and Methods) was used for this and all subsequent studies, except for Figure 2A. ×21,000.

process appeared to be similar to that previously described by Ornberg and Reese (1981). Granules often were seen at the plasma membrane, and granule membrane-plasma membrane fusions also were observed. Asymmetric granules with narrow ends appeared to orient these ends toward the plasma membrane (Fig. 5).

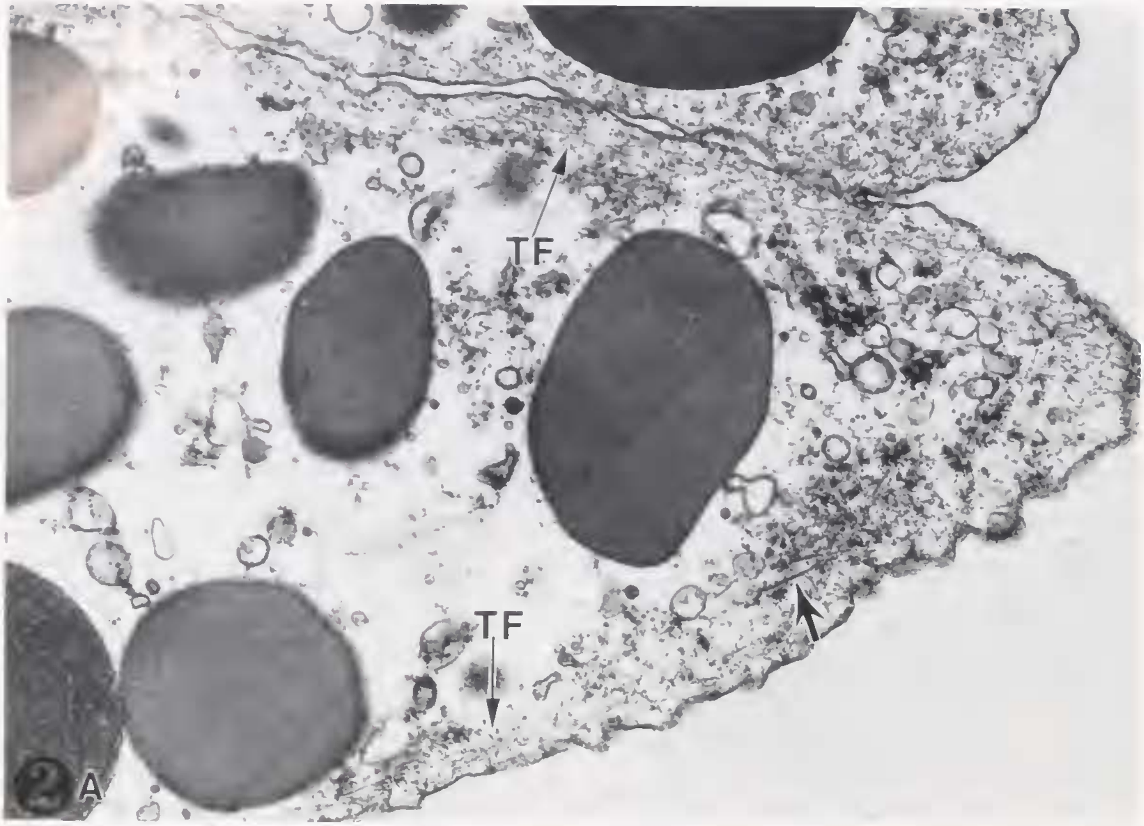

Figure 2A. Native amebocyte fixed by the method of Boyles *et al.* (1985) best reveals the cortical array of thin filaments (TF) which is present throughout the cell. In addition, short 10-nm filaments (▶) are seen among the cortical actin filaments. ×34,500. (Provided by Paula E. Stenberg, unpub. obs.)

This distinctive arrangement was observed in virtually all activated cells. Neither microtubules nor distinct arrays of thin filaments appeared to be associated with sites of exocytosis.

Endotoxin-activated amebocytes

Amebocytes exposed to bacterial endotoxin appeared to be rapidly activated and underwent extensive shape change and degranulation. Subsequently, most cells contained only a small residual population of granules which were located adjacent to the nucleus. In many cells, there was a coalescence of granules and granule membrane fusion was apparent. Unlike amebocytes activated by contact with an endotoxin-free surface (and in which exocytosis occurred following movement of granules to the plasma membrane [Fig. 5]), endotoxin-treated amebocytes appeared to secrete their granules from a more central location within the cell (Fig. 6).

Endotoxin-activated amebocytes were more spherical in shape, with numerous filopodia. Fine filopodia contained bundles of actin filaments, but the overall arrangement of thin filaments was vastly different from either native or surface-activated amebocytes (Fig. 6). Short pieces of thin filaments remained associated with the plasma membrane, and occasionally extended into the cytoplasm as isolated groups of two or three filaments, without apparent organization. A large area of cytoplasm between the plasma membrane and the now centrally located granules and nucleus contained sparse, randomly arranged thin filaments. At the outermost aspect of the centralized granules, there was a loosely organized bundle of actin filaments whose array was similar to the cortical actin seen in native cells (Fig. 6).

The MB surrounded the centralized granules and was located internally to the actin array. In cells that had undergone complete degranulation, the MB was now located adjacent to the nucleus (Fig. 7). The MB appeared to remain intact, and microtubules never were detected in other parts of the cells, under this experimental condition. MB's often were apparently twisted, with microtubules present in both longitudinal and tangential sections of the same cell. This distinct reorganization and central-

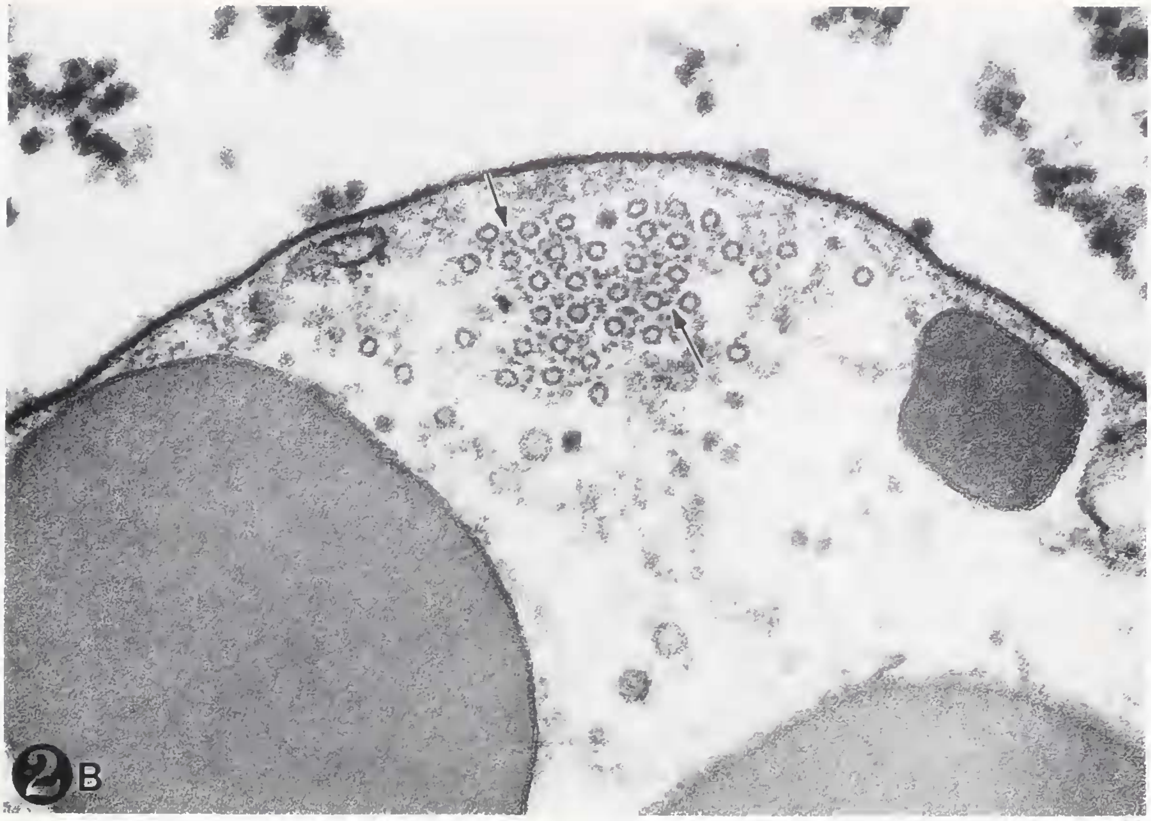

Figure 2B. A higher magnification of the marginal microtubule band (MB) in cross-section. Projections can be seen leading from one microtubule to another (▶). These projections may serve to stabilize the MB, and are likely related to microtubule-associated proteins present in other systems. ×104,000.

ization of the MB only occurred in endotoxin-activated cells.

Nuclear profiles

Amebocyte nuclei of cells studied under all three conditions (native, activated, and endotoxin-activated) had mean axial ratios of 1.67 (native, $n = 30$), 1.62 (activated, $n = 29$) and 1.50 (endotoxin-activated, $n = 30$). These values fit the axial ratios for an oblate ellipsoid, which is the most common nuclear shape of discoid cells (Elias and Hyde, 1983). The slightly smaller mean axial ratios of the nuclei in the endotoxin-activated cells may be due to the change in cell shape from discoid to a more spherical shape. These data were generated to determine if the nuclear profiles changed under the various conditions of the study, as we wished to know whether cytoskeletal changes were secondary to either an increase or decrease in nuclear size. Since nuclear axial ratios did not change appreciably under any of the experimental conditions, these data support our interpretation that the microtubule coil constricts around the nucleus in endotoxin-activated amebocytes.

Discussion

The discoid shape of native amebocytes has been observed with a variety of techniques, including high resolution differential interference contrast (DIC) microscopy (Armstrong, 1979) and electron microscopy (Copeland and Levin, 1985; Levin, 1985b). In this paper, we have presented data which suggest that the two major elements of the amebocyte cytoskeleton appear to act in tandem to maintain the discoid shape of the native amebocyte. The cortical actin array with its link to the plasma membrane, in association with the marginal microtubule band, appears to provide a tension arrangement similar to that proposed for nucleated erythrocytes by Cohen *et al.* (1982).

Following endotoxin-free activation of amebocytes, this arrangement becomes disrupted, and is accompanied by shape change. Shape change of endotoxin-free activated amebocytes is similar to that reported for surface-activated cells by Armstrong (1980). Under both conditions of endotoxin-free activation, cells extend pseudopodia and filopodia. It is interesting to note that while the actin array undergoes reorganization, the mar-

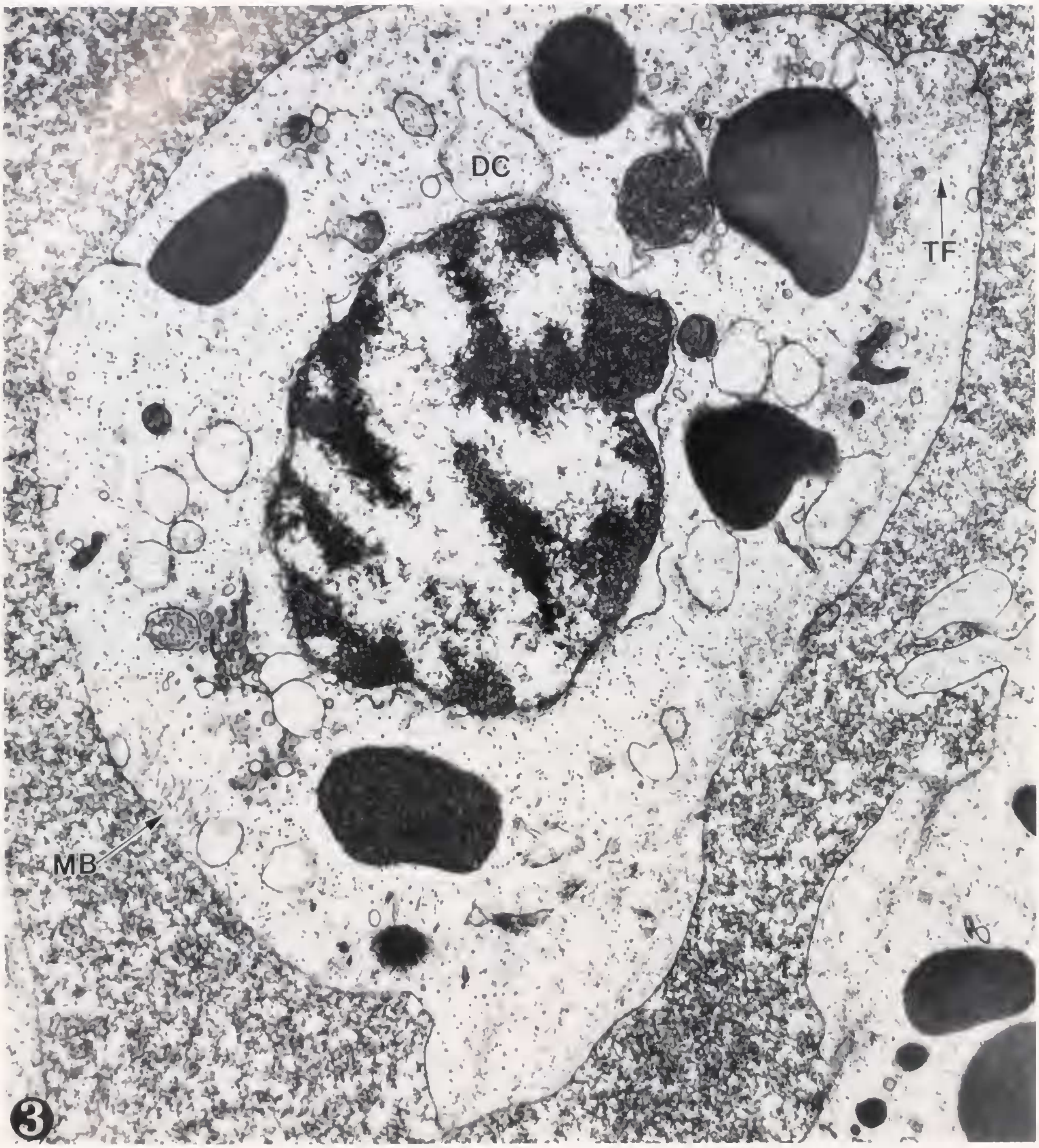

Figure 3. Following endotoxin-free aggregation, activated amebocytes are no longer discoid in shape, and many have secreted the contents of a variable number of their granules. Most cells have pseudopodia, which contain diffuse networks of thin filaments (TF). The MB, still intact, is only present in a tangential section. An area of plasma membrane invagination near a granule (upper left) indicates a likely site of exocytosis. Large irregular dilated cisternae (DC) may represent previous sites of exocytosis. ×21,000.

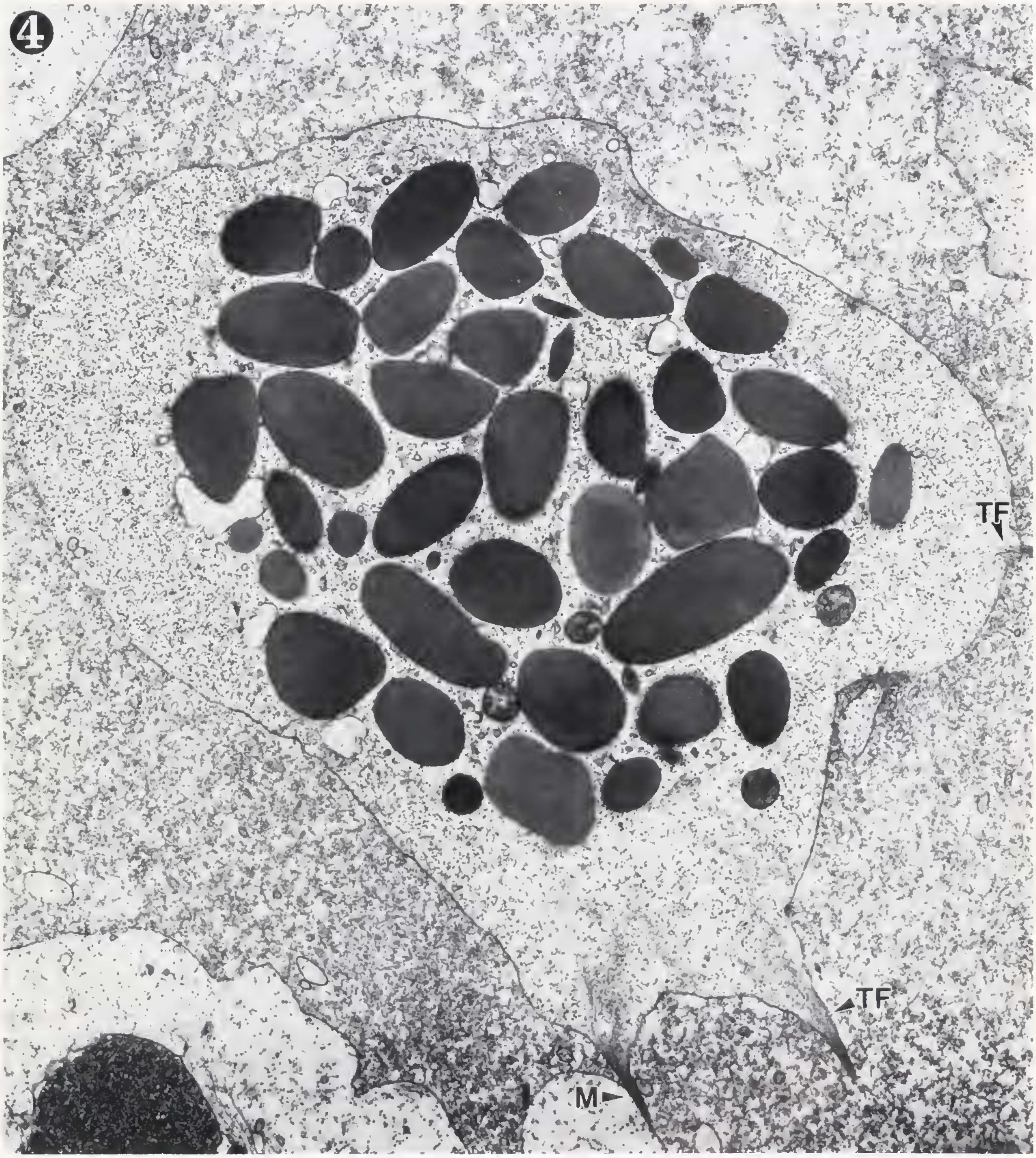

Figure 4. Activated amebocytes, in addition to developing pseudopodia, also produced microspikes (M), which contain fine bundles of thin filaments (TF), some of which remained bundled in the adjacent cytoplasm (▶). It is interesting to note that this cell, which has retained most of its granules, and the cell in Figure 3 were in the same group of surface-activated amebocytes, indicating that cells can undergo exocytosis at different rates. ×14,500.

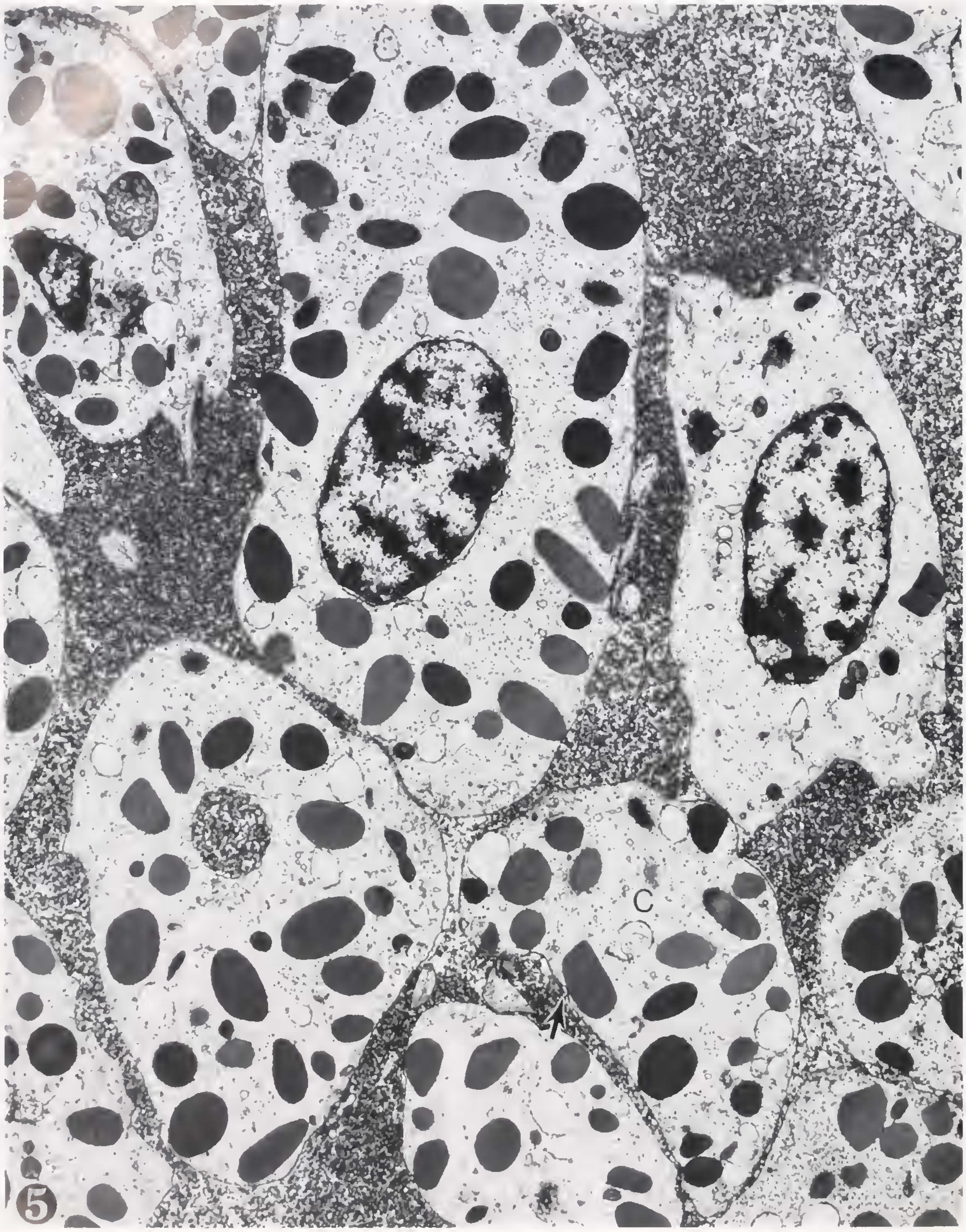

Figure 5. Surface-activated amebocytes demonstrate the distinctive orientation of granules towards the plasma membrane. Note that almost all of the granules are oriented with their "pointed" end toward the plasma membrane. One of the cells (C) (lower center) demonstrates the unusual observation of a granule which is oriented towards the plasma membrane along its broad side. However, an invagination of the plasma membrane appears to contact only one part of this granule (▶). A decrease in the density of cytoplasmic ground substance was apparent throughout the population of activated amebocytes. ×9600.

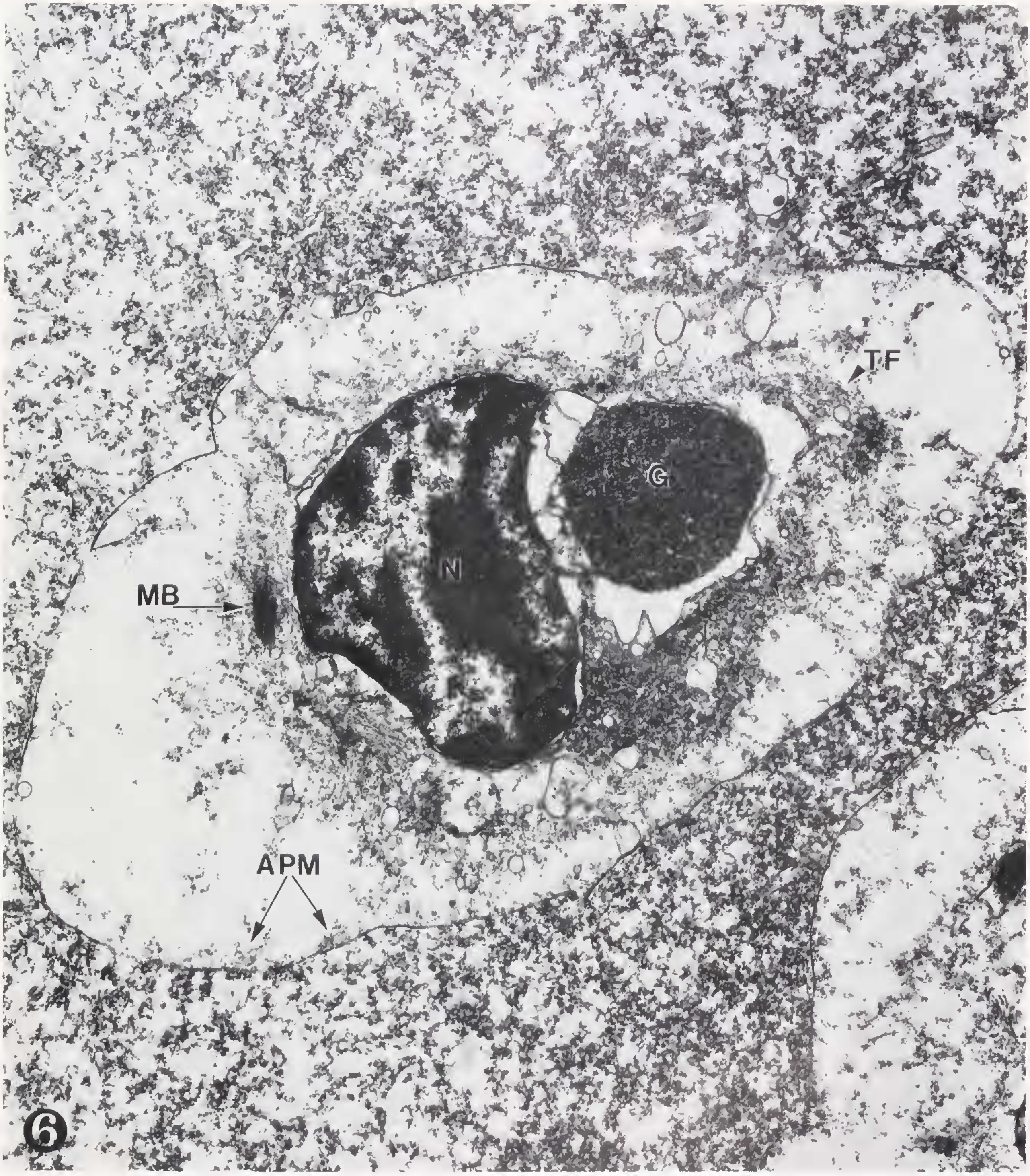

Figure 6. Endotoxin-activated cells usually contained fewer granules, and often were spherical in shape. A fine actin-plasma membrane (APM) network is still in place. However, the majority of the actin (TF), as well as the MB, is now located adjacent to the nucleus (N). A large granule (G) or fusion product of several granules can be seen next to the nucleus. Cytoplasmic ground substance appeared decreased, similar to that observed in surface-activated cells. ×17,200.

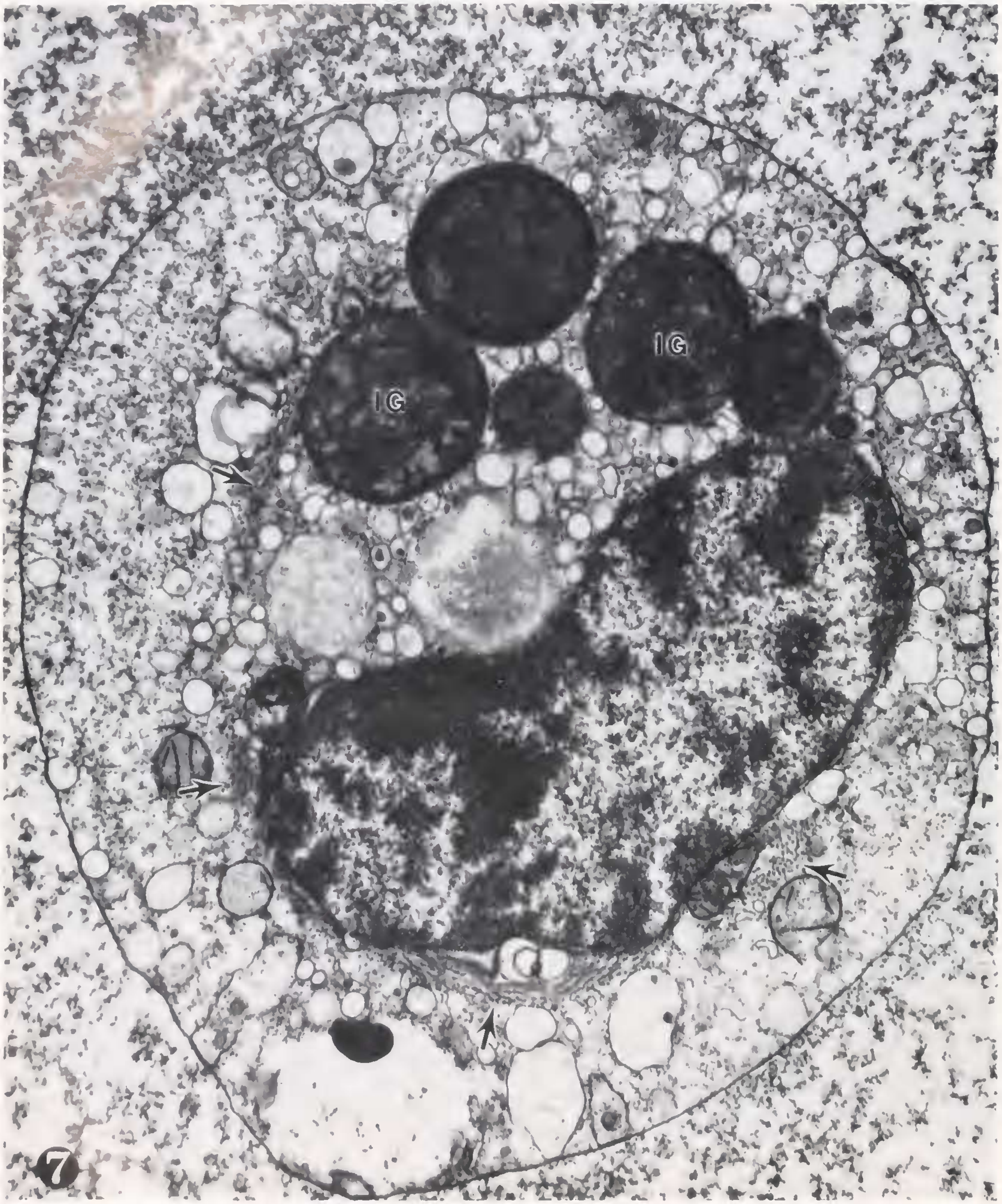

Figure 7. The rearrangement of the cortical actin network in endotoxin-activated cells may exert tension on the marginal band (▶), resulting in both constriction and twisting. This twist is illustrated by the various angles at which microtubules are present (MB). Several centralized immature granules (IG) are also present within the cytoplasm close to the nucleus. The apparent thickening of the granule membranes is likely due to the tangential nature of the section. ×24,800.

ginal band appears to remain quite stable. It may be held together by its projections which are most likely microtubule-associated proteins (Amos, 1977; Kim *et al.*, 1979). While a stable microtubule coil is often found in a variety of nucleated erythrocytes (cells which do not undergo exocytosis), it is not typical for cell systems in which the polarity of secretion is dependent upon a specific microtubule arrangement (Rindler *et al.*, 1987). In activated amebocytes, an entire marginal band may extend into a pseudopodium. We suspect that this is a tension related event in which the pseudopodium is extending within the same plane as the MB. In this case, the actin array reorganizes and extends into the pseudopodium, thus releasing the tension it may have previously exerted on the MB and allowing the MB to expand into the pseudopodium. Rare pieces of individual microtubules were observed within pseudopodia, likely due to a short unwinding of an end of the MB. However, we never observed the complete disassembly of the MB. Some types of invertebrate blood cells are extremely reactive to foreign surfaces (Belamarich, 1976). This is especially true of *Limulus* amebocytes (Armstrong, 1980) and also has been examined in lobster blood cells. However, in lobster blood cells, unlike *Limulus* amebocytes, the marginal microtubule band disassembles when the blood cells are activated by surface contact (Cohen, *et al.* 1983).

The changes detected in endotoxin-treated amebocytes were similar, in part, to the observations of Armstrong and Rickles (1982). They noted that amebocytes treated with endotoxin (1 μg/ml) developed a spherical appearance, a shape consistent with our observations. However, they believed that degranulation only occurred in a small percentage of cells. From our studies, it is apparent that after incubation with endotoxin, amebocytes undergo extensive degranulation as well as shape change. The development of a spherical shape was often accompanied by the presence of fine filopodia. We suggest that the spherical nature of the cells may be due to the vastly increased amount of plasma membrane now present as a result of granule membrane-plasma membrane fusion. This increase in plasma membrane could readily accommodate an increase in the overall volume of the cell, resulting in a large sphere in which thinning of the membrane-associated actin network has occurred. An alternative explanation for the change in plasma membrane-associated actin is that during activation, there is a lowering of the intracellular pH which could result in a net depolymerization of actin (Begg and Rebhun, 1979). This depolymerization in concert with a drop in intracellular pH might also be responsible for the apparent decrease in cytoplasmic ground substance seen in both activated states.

There is also displacement of the cortical actin array and MB to a more centralized location. The rearrangement of the MB to a more centralized location suggests constriction of the band, since it is no longer associated with the periphery of the cell, but now condensed around the nucleus. Furthermore, our determinations of mean caliper diameter, which indicated that the nuclear profiles did not change appreciably under any of our experimental conditions, confirmed that the MB had become constricted around the nucleus. Our observations of twisted marginal bands suggest that the movement of the cortical actin array to a perinuclear location likely exerts tension on the MB, resulting in constriction and twisting. However, not all condensed marginal bands were twisted after endotoxin-induced activation. We speculate that those MB that did twist may represent a less stiff population of microtubules. Marginal bands of dogfish thrombocytes also have been noted to constrict around the cell nucleus during activation and clotting (Shepro *et al.*, 1969).

The interactions between actin filaments and microtubules in normal or activated amebocytes are complex. This is suggested, in part, by their central redistribution and colocalization in the endotoxin-treated cells. If such an association was absent, one would predict that the actin arrays would remain associated with the plasma membrane actin. Actin and tubulin have been shown to colocalize in the fresh water ameba *Reticulomyxa* (Koonce and Schliwa, 1986), and some *in vitro* data suggest that microtubule-associated protein 2 can cross-link actin filaments and microtubules (Griffith and Pollard, 1982). However, there are no reported *in vivo* studies which document linkage either directly between actin and microtubules or via associated linking proteins. Therefore, the nature of the interaction between actin filaments and microtubules in *Limulus* amebocytes remains unknown.

Previous studies have compared *Limulus* amebocytes with anucleate mammalian platelets, with regard to their roles in hemostasis and thrombosis (Levin, 1985a). We also have noted some cytoskeletal similarities between the two cell types. Both amebocytes and human platelets (Fox *et al.*, 1984; Tuszynski *et al.*, 1985; Fox and Phillips, 1986) have an actin-membrane skeleton which appears to link the cytoskeleton with the plasma membrane. The extensive cortical actin array present in *Limulus* amebocytes has been detected in human (Fox and Phillips, 1983; Boyles *et al.*, 1985) and bovine (Tablin, pers. obs.) platelets.

A marginal band of microtubules is present in both cell types; but its properties and potential physiologic roles differ. In both amebocytes and platelets, the marginal microtubule band has been suggested to help maintain the discoid shape of the cells, in conjunction with the actin filament-plasma membrane network (Nachmias. 1980; White *et al.*, 1984; Cohen and Nemhauser, 1985). However, the marginal microtubule band of amebocytes is an extremely stable structure which undergoes very little disruption under a range of stimuli. In contrast, the microtubule coil of both human and bovine platelets appears to be more responsive to physiological stimuli (Debus *et al.*, 1981; White, 1987). Although it appears that the *Limulus* amebocyte cytoskeleton does not play a role in exocytosis, there are suggestions that the human platelet microtubule coil may be important in the centralization of granules prior to secretion (Allen *et al.*, 1979; Stenberg *et al.*, 1984; White and Burris, 1984).

Further studies are needed to fully assess the amebocyte cytoskeleton and its related proteins, and to more completely compare amebocytes with mammalian platelets. We are currently biochemically characterizing amebocyte cytoskeletal components and their potential interactions.

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